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Molecular evolution and radiation of dung beetles in Madagascar

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Abstract

Madagascar is the world's fourth largest island and has a wide range of climates and ecosystems. Environmental diversity combined with long history of isolation (160 Myr) has generated a high level of endemism at different taxonomic levels, making Madagascar one of the hotspots of global biodiversity. Dung beetles, represented by the two tribes of Canthonini and Helictopleurini, exemplify a large insect taxon. Helictopleurini are completely endemic to Madagascar while Canthonini are endemic at generic level. Using data from mitochondrial and nuclear genes, phylogenetic relationships were investigated in a sample of 44 species. The phylogeny for Canthonini consists of several distinct clades, possibly reflecting multiple colonization of Madagascar. The phylogeny does not support the current taxonomy for all genera. The phylogeny for Helictopleurini lacks statistical support at supra-specific level, and genetic divergence among the Helictopleurini species is comparable with that among species within genera in Canthonini. These results suggest that Helictopleurini has undergone rapid speciation and most likely more recently than Canthonini, consistent with the estimated radiation time based on mtDNA mutation rates in insects and with knowledge about the systematics and geographic distribution of dung beetles worldwide. A detailed analysis of sequence composition identified common patterns in Malagasy dung beetles and other insects.

Studies of island radiations of species have played an important part in increasing our understanding of evolutionary and ecological patterns and processes. Some of the largest islands with unique biota have, however, remained poorly studied. Thus, it was not until the 1980s and early 1990s that comprehensive investigations were initiated on the biota of the Indian Ocean islands, including Madagascar (Kull, 1996). Madagascar, the world's fourth largest island, can be considered a mini-continent (Yoder and Yang, 2004; references therein) for its large area (586 000 km^2) and varied assortment of climates and ecosystems. This variety together with long-term geographic isolation (160 Myr) has contributed to an exceptionally high level of endemism at different taxonomic levels (Paulian, 1987; Yoder and Yang, 2004).

The global dung beetle (Scarabaeidae) fauna is divided into 12 tribes (Cambefort, 1991). Two tribes, Canthonini and Dichotomini, are widespread with principal generic richness in the southern continents (Afrotropical, Neotropical, Madagascar and Australia/New Guinea). These tribes alone contain more than half of the world's Scarabaeidae genera (Davis et al., 2002). The Malagasy Canthonini, endemic at generic level, includes 13 genera and $\approx$170 described species. The tribes Phanaeini, Eucraniini, Eurysternini and Helictopleurini have geographic distributions that are restricted, respectively, to the Americas (the first three) and Madagascar (Helictopleurini) (Davis et al., 2002). Helictopleurini is represented in Madagascar by two genera, one monotypic and the other one with $\approx$60 described species. The taxonomy of Malagasy Canthonini and Helictopleurini was revised in the fifties and sixties by Paulian and Lebis (Lebis, 1953; Paulian and Lebis, 1960). Subsequently, new species have been assigned to the old and some new genera (Paulian, 1975, 1986; Paulian and Cambefort, 1991). Unfortunately, misinterpretation of some morphological characters, such as the shape of the male fore and hind legs

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and characters in male genitalia have produced incongruences in the taxonomic classification. Currently a more comprehensive taxonomic review of the two tribes is in progress (Montreuil, 2003a,b, 2004, 2005a,b,c,d) and the first ecological and biological studies have been completed (Koivulehto, 2004; Viljanen, 2004).

A pervasive challenge in molecular systematics is the incongruence of phylogenies based on different sets of genes, and therefore analyses based on two or more genes have become increasingly popular. Multigene approaches have been used to address the evolution of plants, animals, algae and eukaryotes (Gontcharov et al., 2004, references therein). A related question is which genes are most appropriate to infer phylogenetic relationships. Mitochondrial genes have been the most commonly used source of data in studies of insect molecular phylogeny and phylogeography (Caterino et al., 2000; Avise, 2004). They are assumed to vary in a neutral manner and their patterns of nucleotide variation have been used to infer the evolutionary histories of closely related species (Rand et al., 1994). Mitochondrial genes are generally easy to amplify and specific primers are available for many species (Simon et al., 1994), they lack introns, which are problematic in phylogenetic analyses, and they are maternally (clonally) inherited. Last but not least, these genes are estimated to evolve two to nine times faster than nuclear protein-coding genes in insects (DeSalle et al., 1987; Moriyama and Powell, 1997; Monteiro and Pierce, 2001).

Although mitochondrial genes are the most commonly used genes to resolve relationships at the species level, nuclear genes provide more power to resolve relationships at higher taxonomic levels (Hillis and Dixon, 1991). Therefore, it is now common practice among insect molecular systematists to combine mitochondrial and nuclear genes. The principle of total evidence (Kluge, 1989) assumes that all molecular evidence possesses equal value in discriminating among phylogenetic hypotheses. Employing this principle is not to say that all information is equally discriminating, but this cannot be known *a priori*. Furthermore, not to be underestimated are the different evolutionary constraints characterizing the two classes of genes, which introduce additional problems in data analysis. The common practice of concatenating sequences obtained from different genes may mix phylogenetic signals arising from different evolutionary histories (owing to recombination, introgression, and other processes), and it may lead to complications in the analyses due to missing data for some species (Wiens, 1998; Kearney, 2002). Ideally, the combined analysis should allow different sets of model parameters to be used for different genes. The Bayesian phylogenetic inference is able to combine information from different data partitions evolving under different stochastic evolutionary models and thus allows the analysis of heterogeneous data. Bayesian inference of phylogenies is based upon the posterior probability distribution of trees, which is the probability of a particular tree conditioned on the observed parameters.

Alternatively, the analysis should make as few *a priori* assumptions as possible, thus reducing constraints on possible transformations and inferring characters *a posteriori*. The latter requirement is fulfilled by the program POY 3.0.11 (Phylogeny Reconstruction via Optimization of DNA and other data) (Wheeler et al., 1996–2003), which tests phylogenetic hierarchies with the broadest possible set of evidence and employs the total evidence principle. It also combines the two historically disconnected processes of multiple alignment and cladogram searching into one step using the dynamic homology approach.

Here we use sequence information from mitochondrial and ribosomal nuclear genes and the principle of total evidence to reconstruct the molecular phylogeny of Malagasy dung beetles and to assess whether their origin in Madagascar can be associated with a rapid radiation. The phylogenetic relationships are analyzed using both the above-mentioned approaches, one based on the absence of *a priori* assumptions and the second one using different evolutionary models appropriately estimated to fit different partitions of data. Additionally, we examine evolutionary trends and gene substitution patterns in Malagasy dung beetles, identifying similarities with other insect taxa.

Materials and methods

Taxa

An intensive trapping program across Madagascar has yielded a large fraction of Helictopleurini (Coprinae) and Canthonini (Scarabaeinae) species. The specimens were collected between 2002 and 2004 by pitfall trapping, baited with fish or lemur dung and subsequently preserved in 90% ethanol. Sequence data from two nuclear (18S, 28S) and five mitochondrial genes (16S, 12S, cytochrome oxidase subunit 1, cytochrome oxidase *b* and cytochrome oxidase subunit II) were analyzed for 17 species of Helictopleurini and 27 species of Canthonini (Appendix 1). The sequence data consist of about 5 kb of nucleotide information, mainly present in the mitochondrion. For cytochrome oxidase subunit 1 and cytochrome oxidase *b*, two contiguous regions were amplified and sequenced. Sequences were submitted to GenBank (Appendix 2). For gene sequences the following abbreviations are used throughout this paper: 16S—16SrRNA; 12S—12SrRNA; COITH and COIJN—cytochrome oxidase subunit 1; *Cytb*B and *Cytb*K—cytochrome oxidase *b*; COII—cytochrome oxidase subunit II; 18S—18S rRNA; and 28S—28S rRNA.

DNA extraction and sequencing

Specimens were pressed firmly between blotting paper to remove excess ethanol. We used the whole body of small insects and a leg or two of larger individuals for DNA extraction. Whenever possible the gut and the head were removed to avoid contamination. DNA extraction was performed using the Nucleo spin tissue extraction kit (Mackerey-Nagel, Düren, Germany), with O/N incubation at 56 °C. Twenty to 30 ng of genomic DNA was amplified in polymerase chain reactions (PCR) consisting of 1 μM of each primer, 200 μM of each of the dNTPs, 2.5 mM of $MgCl_2$, 20 ng of bovine serum albumin and 0.1 U of Taq Polymerase (Fermentas Life Sciences, Finland). All amplifications were performed in 20 μL final volumes using MBS 0.2G thermal cycler (Hy-Baid, Thermo Electronic Corp., Waltham, MA). PCR cycling conditions were as follows: denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, Tm (depending on the locus) for 1 min and 72 °C for 1.5 min. A final extension step of 15 min at 72 °C was included to stabilize the polymerase. PCR primers and their bibliographic sources are listed in Table 1. PCR products were purified using GFX purifying kit (GE Healthcare Europe, Germany) and double-strand sequenced using Big Dye terminator chemistry on Megabace 1000 automated sequencer (GE Healthcare). Sequence assembly and editing were done using DNA Star software package (Lasergene, DNA Star Inc., Wisconsin, USA).

Sequence analyses

The entropy-based index of substitution saturation (I_{ss}) was used to test whether the sequences showed signs of saturation in the nucleotide mutations (Xia and Xie, 2001). I_{SS} is compared with the critical value $I_{SS.C}$, at which the sequences fail to reflect the true phylogenetic signal due to high level of saturation. If I_{SS} is significantly smaller than $I_{SS.C}$, the sequence does not show significant saturation, while it is saturated if I_{SS} is not significantly smaller than the critical value (Xia et al., 2003). Sequence saturation was calculated for different codon positions for the protein-coding genes. The nucleotide composition at each codon position in each gene region was calculated and χ^2 statistics were used to test for homogeneity of base frequencies among taxa.

Transformation rates were calculated for individual mitochondrial genes to investigate the transition/transversion ratios. For the protein-coding genes the transition/transversion ratios were calculated at different codon positions, while for the non-coding genes (12S and 16S) a single transformation rate was calculated. Numbers of transitions and transversions were calculated for single genes. Within the two classes of transformations the most common mutation(s) were identified.

Sequence polymorphism was calculated for single genes as the number of variable sites (*VS*), which include parsimony informative and singleton sites. The level of polymorphism was compared between tribes. The level of polymorphism was also compared among clusters identified by the Canthonini phylogeny and the genus *Helictopleurus*. Because the different numbers of species in different taxa would affect the comparisons, the original species number in different groups was rarefied to the same number (with appropriate replication). Genetic distances between pairs of species were calculated within tribes and within clades for each gene.

Table 1
PCR primers and bibliographic sources for the loci used in this paper. For locus abbreviations see Materials and methods

Locus (length in bp)	PCR and sequencing primers (5′–3′)	Source
16S (354–362)	16Sf (luisa) ATGTCTTTTTGAKWATAATWTAAAG 16Sr (luisa) ACGCTGTTATCCCTAAGGTAATTT	Present paper
12S (360–372)	SR-J-14233:AAGAGCGACGGGCGATGTGT SR-N-14588: AAACTAGGATTAGATACCCTATTAT	(Kergoat et al., 2004)
CytbB (333)	CB3: GAGGAGCAACTGTAATTACTAA CB4: AAAAGAAA(AG)TATCATTCAGGTTGAAT	(Balke et al., 2004)
CytbK (697)	Cp1: GATGATGAAATTTTGGATC CB-N-11367: ATTACACCTCCTAATTTATTAGGAAT	(Kergoat et al., 2004)
COI JN (420)	C1-J-1751: GGATCACCTGATATAGCATTCCC C1-N-2191: CCCGGTAAAATTAAAATATAAACTTC	(Simon et al., 1994)
COITH (509)	TONYA: GAAGTTTATATTTTAATTTTACCGGG HOBBES: AAATGTTGNGGRAAAAATGTTA	(Kergoat et al., 2004)
COII (695)	COIIF-leu: TCTAATATGGCAGATTAGTGC COIIR-lys: GAGACCAGTACTTGCTTTCAGTCATC	(Whiting, 2002)
18S (830)	18Sai CCTGAGAAACGGCTACCACATC 18Sbi GAGTCTCGTTCGTTATCGGA 18Sa0.7 ATTAAAGTTGTTGCGGTT 18Sb3.0 GACGGTCCAACAATTTCACC	(Whiting et al., 1997)
28S (332)	28sa GACCCGTCTTGAAACACGGA 28sb TCGGAAGGAACCAGCTACTA	(Whiting et al., 1997)

Hierarchical likelihood ratio tests were calculated for the single gene partitions using Model Test 3.04 (Posada and Crandall, 1998) to determine the best-fit substitution model of nucleotide evolution out of the set of 56 models implemented in the program.

Phylogenetic analyses

As an initial step different partitions represented by single genes were used to reconstruct gene trees, while phylogenetic relationships were ultimately inferred based on total evidence trees for both tribes. Single gene trees and total evidence trees were obtained using both maximum parsimony and Bayesian analysis. For the parsimony analysis, trees were constructed by combining all available evidence and searching for the simplest (most parsimonious) explanation for character variation, using POY (Wheeler et al., 1996–2003). Molecular data were optimized using the "dynamic homology" procedure, for which data are not encoded (or aligned) in a fixed character matrix prior to the phylogenetic analysis. Tree search command executed in POY included random sequence addition followed by a fast parallelized tree-building step and TBR branch swapping. The number of replicates was 100 with five additional sequences in the building phase of a single replicate. We used equal weighting of gaps and transformations because this has been shown to yield the most congruent results (Schulmeister et al., 2002). Calculations were speeded up by stopping random cladogram building after 20 equally short trees had been found in at least 50 replicates. Branch-swapping was performed for all the trees with length within 2% of the shortest one. Jackknife values (100 replicates) were used as a measure of node support. We present the best tree obtained with 100 random addition replicates and including the two shortest trees in each replicate.

Prior to the Bayesian analysis sequences were aligned using Clustal W (Thompson et al., 1994) in Bioedit version 6.0.7. The Bayesian analysis was performed with the program MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). One cold and three incrementally heated Markov Chain Monte Carlo chains were run for between 1 and 18 million cycles, with trees sampled each 1000th generation, using a random tree as a starting point and a temperature parameter of 0.2 (the default in MrBayes). For each data set, MCMC runs were repeated twice to eliminate spurious results. The first 25% of the trees were discarded and the remaining trees were used to construct consensus trees. The evolutionary models obtained with Modeltest were used to set the priors of the MCMC runs.

Parascatonomus penicillatus (Harold, 1879) was used as an outgroup for Helictopleurini. This species belongs to the tribe Onthophagini, the sister tribe of Oniticellini (Villalba et al., 2002), in which the genus *Helictopleurus* was originally placed (d'Orbigny, 1915). For Canthonini an appropriate outgroup representing putative African or Australian relatives of Malagasy Canthonini was not available either as a sequence or a tissue sample, and we were forced to choose an outgroup among the sequences we produced. Our choice was the genus *Aleiantus,* because it has been considered to be the most basal among the genera we analyzed based on morphology (O. Montreuil, pers. comm.).

Results

Sequence analyses

The mitochondrial and nuclear gene sequences used in this study did not involve indels. Hence the alignment used in the Bayesian analysis did not require much manual adjustment following the alignment of the sequences with the program Clustal W (Thompson et al., 1994). The nuclear loci did not show significant saturation, while all the mitochondrial loci showed saturation at the third codon position (Table 2). Cytochrome subunit 1 (COITH and COIJN) and *Cytb*K were the least saturated loci at the first two codon positions in both tribes.

Base composition was significantly AT-biased in the mitochondrial genes regardless of whether the codon positions were analyzed separately or together. The nuclear genes showed no bias and the nucleotide composition was evenly distributed among the four nucleotides (Appendix 3). The most frequent transformation in the mitochondrial genes was the AT transversion, followed by AG and CT transitions. The latter two are equally frequent in the genes examined here (Fig. 1). Calculating a single transition/transversion ratio, which is common practice in molecular systematic studies, would entirely obscure these patterns. Strong AT bias and small transition/transversion ratios are

Table 2
Sequence saturation at different codon positions for the protein-coding mitochondrial and ribosomal genes. The *P*-values give the probability of the base not being saturated

Tribe	Locus	1st codon	2nd codon	3rd codon
Canthonini	Cytb B	0.01	0.09	0.28
	Cytb K	0.04	0.08	0.38
	COI JN	0.04	< 0.01	0.50
	COI TH	< 0.01	0.04	0.44
	COII	0.07	0.06	0.30
	18S	0.01	0.01	0.01
	28S	0.04	0.04	0.04
Helictopleurini	Cytb B	0.13	0.01	0.30
	Cytb K	0.03	0.09	0.54
	COI TH	< 0.01	0.02	0.43
	COII	0.07	< 0.01	0.30
	18S	0.07	0.07	0.07
	28S	0.02	0.01	< 0.01

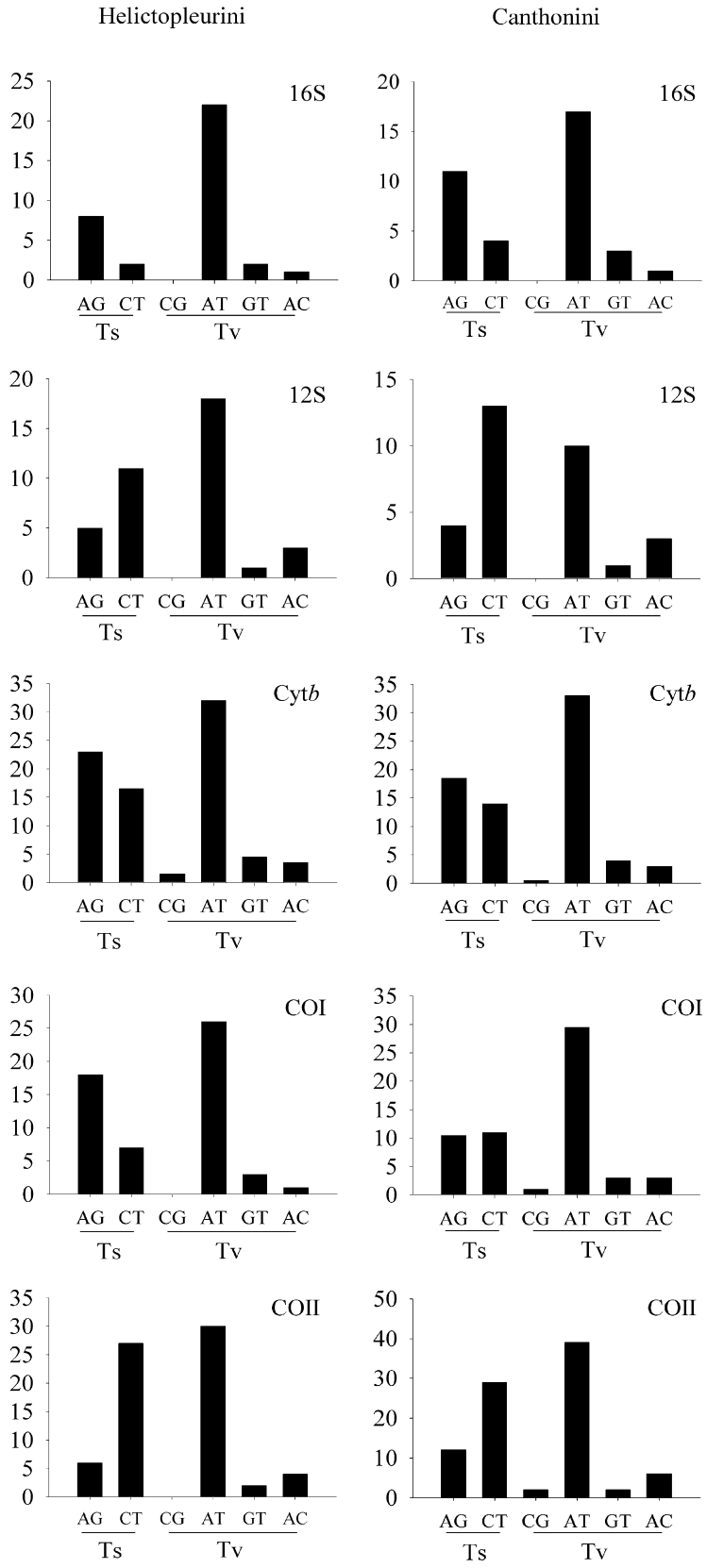

Fig. 1. Transformations in the mitochondrial loci in Canthonini and Helictopleurini. Note the different scales on the vertical axes.

common to all mitochondrial genes in the two tribes. Further analysis of the transformations at different codon positions revealed a trend of higher transversions than transitions in both tribes (Fig. 5).

Sequence polymorphism was compared between the tribes and among the clades (defined below, Fig. 2A,B). Bias introduced by dissimilar numbers of species was eliminated by rarefying the number of species in all the groups examined to the same number. For the sequences that were saturated at the third codon position (protein--coding mitochondrial genes), we calculated polymorphism for the first two codon positions only. In Helictopleurini, the level of polymorphism was higher than in the Canthonini clades in three mitochondrial sequences (16S, 12S and *Cytb*B), but lower than in the most diverse Canthonini clade (*Arachnodes*) in the remaining sequences (Table 3). In Canthonini, the *Sphaerocanthon* clade was clearly the least variable clade (Table 3).

Pair-wise genetic distances show the smallest average distance in Helictopleurini, intermediate within clades in Canthonini, and the largest average distance among pairs of species representing different clades of Canthonini (Fig. 3, Table 3). This is especially clear for the nuclear genes, which showed no saturation, but also for the two mitochondrial genes (COITH and *Cytb*K) that did not show significant saturation in the first two (COITH) or in the first (*Cytb*K) codon position (Table 2). The third codon position was excluded in the calculation of the pair-wise genetic distances in protein-coding mitochondrial genes.

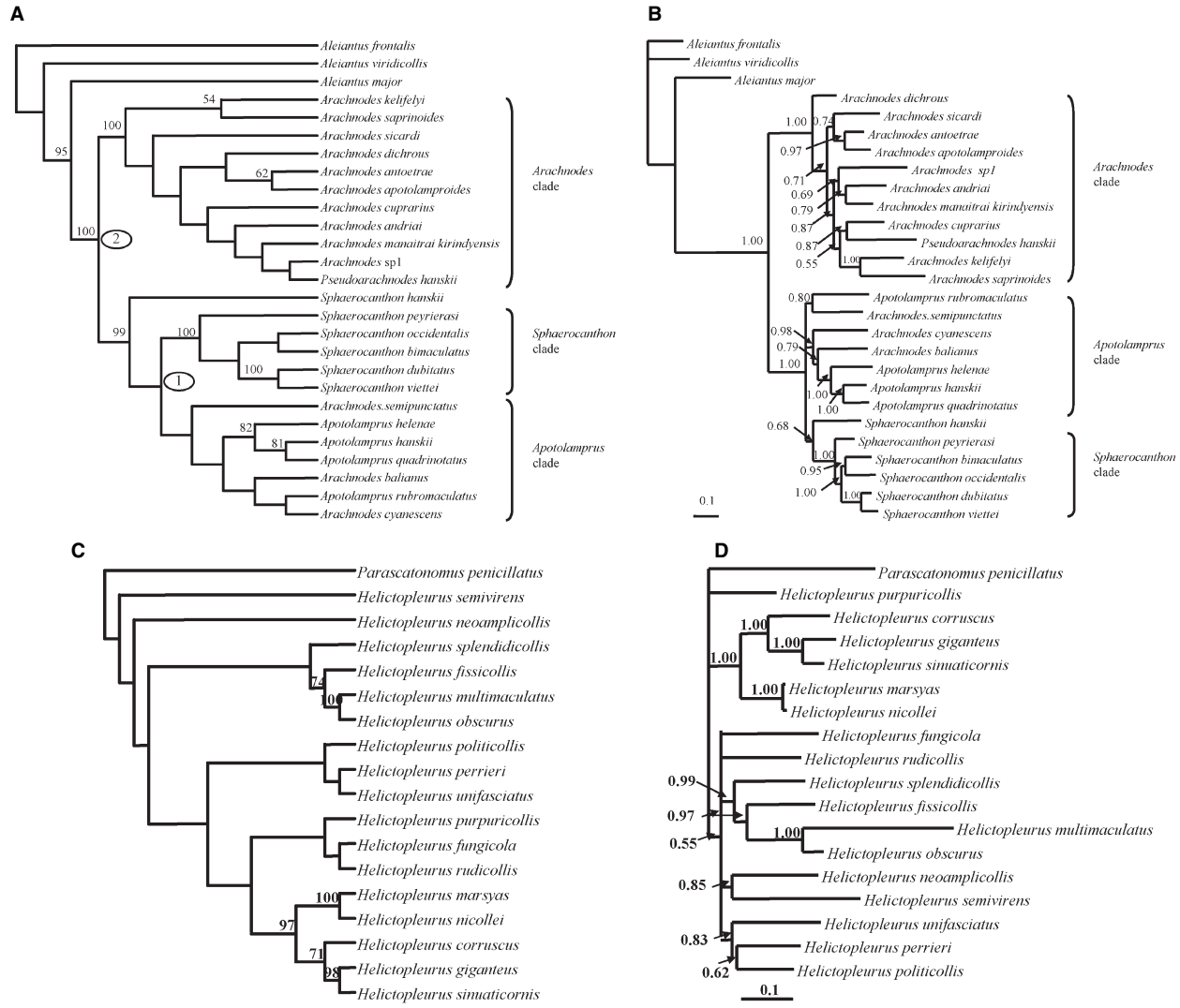

Fig. 2. Total evidence strict consensus parsimony (A, C) and consensus Bayesian trees (B, D) for Canthonini and Helictopleurini, respectively. The trees are based on data for two nuclear and five mitochondrial loci. Jackknife support values and posterior probabilities are shown above the branches with cutoff value of 50%. In the Canthonini parsimony tree (A), the nodes for which divergence times between clades were calculated are indicated with circled numbers: 1 is 10.4 Myr and 2 is 14.2 Myr.

Table 3
Sequence polymorphism among the clades identified in the Canthonini phylogeny and the genus *Helictopleurus*. VS% denotes the percentage of variable sites, "–" denotes no sequence available for the specific locus/genus, and PDM gives the pair-wise genetic distance calculated within clade/genus. VS% for mitochondrial genes are calculated excluding the third codon position, except for 16S and 12S. VS% was calculated by rarefaction for 5 species. The standard deviation was mostly < 0.02.

	Arachnodes clade		*Apotolamprus* clade		*Sphaerocanthon* clade		*Helictopleurus*	
Locus	VS%	PDM	VS%	PDM	VS%	PDM	VS%	PDM
16S	14.8	0.078	16.9	0.073	7.5	0.036	21.2	0.087
12S	13.2	0.054	15.5	0.065	10.3	0.034	21.5	0.115
Cytb B	8.95	0.219	11.4	0.213	5.9	0.161	20.1	0.161
Cytb K	13.1	0.150	11	0.058	8.6	0.042	13.5	0.073
COIJN	9.2	0.183	6.1	0.152	3.6	0.105	–	–
COITH	6.3	0.039	4.8	0.026	1.2	0.006	4.4	0.024
COII	9.8	0.149	5.1	0.112	5.6	0.024	10.4	0.156
18S	0.6	0.001	0.002	0.002	0	0	0.5	0.001
28S	2.8	0.02	0.01	0.01	0.01	0.01	0.9	0.003

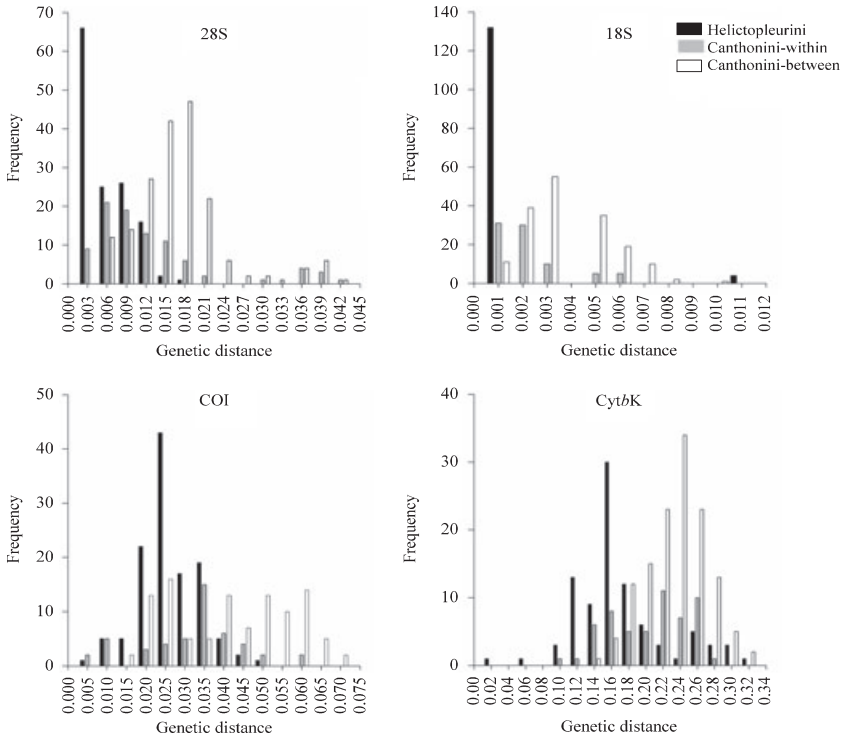

Fig. 3. Frequency distributions of genetic distances between pairs of species for two nuclear (18S and 28S) and two mitochondrial (COITH and *Cytb*K) genes. Results are shown separately for pairs of species in Helictopleurini and within and between the Canthonini clades.

Different DNA regions fitted best different models of nucleotide substitution. Levels of model complexity were similar across mitochondrial genes, where the best model was the six-parameter general-time reversible model of nucleotide substitution (GTR + I + G; Rodriguez et al., 1990). The basic GTR model allows for unequal base frequencies, four classes of transversions and two classes of transitions. Moreover, this model also accounts for the proportion of invariable sites (I) and among-site rate variation (G) (Appendix 4). Among the mitochondrial genes, 12S (Canthonini and Helictopleurini) and 16S (Helictopleurini) are better explained by simpler models than GTR + I + G, implying equal base frequencies (F81 + G) or only some classes of transitions (e.g., TrN; Tamura and Nei, 1993). The nuclear genes obey models of nucleotide evolution with

equal base frequencies (e.g., JC; Jukes and Cantor, 1969) or models in which the rate of transitional nucleotide substitution per site per year (α) is assumed to be different from that of transversional nucleotide substitution (2β) (e.g., K80 + I; Kimura, 1980).

Phylogenetic analyses

Trees obtained with different single genes showed inconsistent clustering of species and in some cases produced conflicting results (data not shown). In Canthonini, *Aleiantus* and *Sphaerocanthon* clustered in all trees as expected based on current taxonomy, but *Sphaerocanthon* did not form a monophyletic group in all gene trees. *Arachnodes* is never monophyletic and shows paraphyly with *Apotolamprus* in all gene trees. The 12S gene tree shows many polytomies and genera are not clustered according to current taxonomy. The nuclear genes do not resolve polytomies at the species level.

In Helictopleurini, the mitochondrial genes yield an inconsistent clustering of species; the only stable cluster for different genes is the *giganteus* group (*Helictopleurus corruscus*, *H. giganteus*, *H. sinuaticornis*, *H. marsyas* and *H. nicollei*). Species in this group share similar morphological features, including shape, surface structure and large size. The nuclear genes gave completely unresolved trees.

The total evidence trees, obtained by combining information from the mitochondrial and nuclear genes, produced a statistically supported phylogeny for Canthonini. The results from parsimony and Bayesian analyses were highly comparable, though the Bayesian analysis yielded a larger number of supported nodes than the parsimony tree (Fig. 2A,B). The genera *Arachnodes* and *Apotolamprus* are polyphyletic. The genus *Sphaerocanthon* is monophyletic in the Bayesian analysis, whereas it is paraphyletic in the parsimony tree (Fig. 2A,B).

The phylogeny obtained for Helictopleurini is well resolved and the trees obtained with parsimony and Bayesian analysis are consistent (Fig. 2C,D)

Discussion

The main results of this paper can be summarized as follows. Mitochondrial and nuclear genes have different nucleotide composition with high AT richness in mtDNA, and the two classes of genes show patterns that are consistent with different evolutionary models. A six-parameter general-time reversible model of nucleotide substitution explains most of the variation in the mitochondrial genes, whereas patterns in the nuclear genes can be explained by simpler models assuming equal base frequencies and/or allowing for only some classes of transitions. Mitochondrial genes are mostly saturated at the third codon position, while the nuclear genes do not show saturation. Phylogenetic analyses revealed polyphyletic genera in Canthonini, and the phylogeny was statistically supported. In contrast, the phylogeny for Helictopleurini lacks statistical support at the deeper nodes, while it is supported at the species level. This is especially true for the Bayesian phylogenetic tree. The difference between the two tribes will be related below to the likely difference in their evolutionary history and respective radiations in Madagascar.

Sequence analyses

Our results indicate that cytochrome oxidase subunit 1 (COITH and COIJN) and cytochrome oxidase *b* (*Cytb*K) are well suited for systematic studies of dung beetles, being highly polymorphic without being affected by significant sequence saturation. Nonetheless, the total evidence approach has the best explanatory power regardless of the method used to infer phylogenetic relationships.

High asymmetry of base substitution rates characterizes all mitochondrial loci. This result is not surprising, because asymmetrical transformation patterns are common in insect mtDNA (Simon et al., 1994; Lin et al., 2004). Data for 19 previous comprehensive studies with mostly 20–50 taxa per study show that the AT transformation is the most common in insects, including Malagasy dung beetles (Fig. 4; full data in Appendix 5). On the other hand, there appears to be a difference in the transition/transversion (Ts/Tv) ratio, dung beetles showing a lower ratio than the previous insect studies. A paired *t*-test indicates that the difference is not significant for COI while it is significant for COII ($P < 0.02$). The difference in the Ts/Tv ratios among the loci could be due to different biases in the type of character changes. Nucleotide changes in protein-coding regions are predominantly at the third codon position and may be biased as a consequence of the preponderance of synonymous changes and natural selection maintaining codon usage. High AT richness in mtDNA leads to relatively high proportion of transversions (mostly those involving A and T) and thus keeps the skew in the Ts/Tv ratio relatively low. In 16S, the skew towards A/T transversions is even stronger, possibly because this sequence is not constrained by codon usage. Selection due to secondary structure interactions may also be involved.

The highly skewed representation of different transformations in mitochondrial genes may induce a high level of homoplasy that is not easily corrected by a simple transition/transversion weighting scheme. In fact, the skewed representation of transformations reduce the number of actual states that can occur at a nucleotide site from four (A, C, G, T) to only two (A,

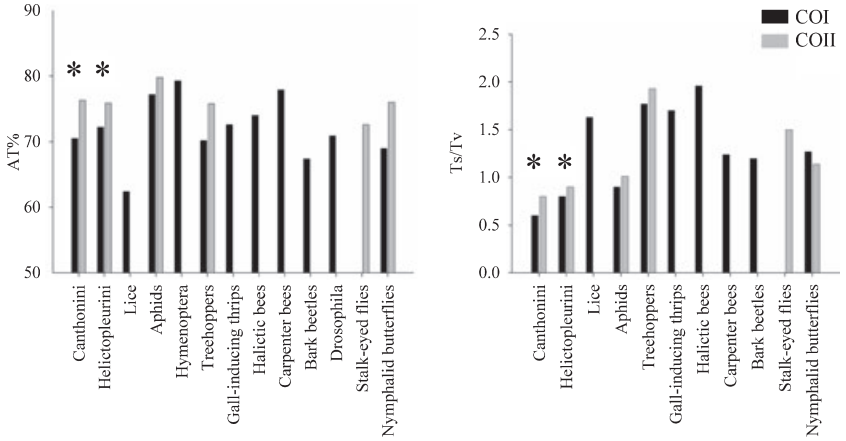

Fig. 4. Comparison of AT richness (A) and transition/transversion ratios (Ts/Tv) (B) for the COI and COII genes between Malagasy dung beetles (*) and other groups of insects (full data in Appendix 6).

T). It is clear that mtDNA can hide strong bias in transition/transversion ratios, which does not follow a common pattern in all insects. This and strong AT bias may explain why mitochondrial genes may perform poorly in analyses based on *a priori* assumptions, while *a posteriori* weighting (in parsimony) or complex models that account for complex base substitution patterns may alleviate these problems (Johnson et al., 2003; Lin et al., 2004). This conclusion is supported by the fact that different data sets resulted in different best-fit models of nucleotide substitution, suggesting that blind model selection may bias phylogenetic inferences. Moreover, while the use of multiple genes increases the power to detect true phylogenies, one has to be aware that different genes from the same genomic region (e.g., mitochondrion) may evolve under different models of evolution and be affected by different substitution patterns. In such cases the use of as few *a priori* assumptions as possible and/or the use of different evolutionary models appropriately estimated for different partitions is the best course of action to obtain reliable results.

Turning to the level of polymorphism among the clades in Canthonini, *Arachnodes* is the most variable one. This genus is paraphyletic with *Apotolamprus,* shows much variation in morphological traits and is in need of a taxonomic revision, which is likely to result in several genera (O. Montreuil, pers. comm.). The level of polymorphism in Helictopleurini was similar to that within individual clades in Canthonini, though there was some variation among the genes. The analysis of pair-wise genetic distances within clades produced a comparable result. Repeating the calculations with the third codon position included, which is commonly saturated in mitochondrial genes, Helictopleurini showed for some genes greater divergence among the species than the entire tribe Canthonini (data not shown). We conclude that one should be aware that patterns of nucleotide saturation may affect inferences about molecular evolution as well as phylogenetic analyses.

The distribution of pair-wise genetic distances in Helictopleurini reveals a small number of very closely related species or subspecific taxa (Fig. 3B). These cases involve taxonomically well-supported species pairs that cluster together in the phylogeny: *H. giganteus* and *H. sinuaticornis*, *H. marsyas* and *H. nicollei*, and *H. multimaculatus* and *H. obscurus*.

Phylogenetic analyses

Comparison of results obtained with different methods is a widespread practice in phylogenetic studies, and any differences in such comparisons are often taken as evidence of long-branch attraction or unresolved phylogeny. Adding more data can resolve such discordances if the additional data are of the same kind (Bergsten, 2005), but in practice adding more data generally means adding data sets with different properties. Recent studies have demonstrated that Bayesian trees estimated from DNA sequences are the most accurate, followed by maximum likelihood and parsimony trees (Ronquist, 2004; Hall, 2005). However, Bayesian analyses seem to be liable to produce erroneous trees when a wrong model is used for the inference (Erixon et al., 2003; Suzuki et al., 2002), and empirical observations suggest that simple evolutionary models tend to be associated with overestimation of posterior probabilities more often than complex models (Nylander et al., 2004).

Taking into account the pros and cons of each method, the most reasonable approach is to use both parsimony and Bayesian estimation and interpret any topological differences as real uncertainty (Hall, 2005). In the present study, the topologies obtained with parsimony and Bayesian analysis for Helictopleurini

and Canthonini are highly comparable. The only noticeable discordance concerns the genus *Sphaerocanthon*, which is monophyletic in the Bayesian tree and paraphyletic in the parsimony tree (Fig. 2A,B). This discordance is not surprising because different genes gave incongruent topologies and did not consistently imply monophyly for this genus. Alternatively, the incongruence could be due to the greater tendency of the parsimony analysis to group long branches together (rightfully or wrongfully) (Pol and Siddall, 2001; Swofford et al., 2001).

Incongruities between the topologies based on different genes were observed in both methods, suggesting that divergence in some loci is greater, while at other loci the species may share a more recent ancestor. This is not surprising: gene trees do not always match with species trees, and the discordances between genealogies may simply reflect differences in expected coalescence times among the loci (Tajima, 1983; Pamilo and Nei, 1988). The short length of individual gene fragments (small number of informative sites) may also be a limiting factor for the performance of single genes.

Considering the phylogenies based on total evidence, the patterns are quite different for the two tribes. In Canthonini, there are polyphyletic genera, suggesting that the current taxonomic classification is incorrect. The current taxonomy is based on morphological characters and due to the work of two authors (Lebis, 1953; Paulian, 1975, 1986). In the course of a revision of Canthonini, many species as well genera were discovered to be in need of synonymization, whereas other species have been classified in wrong genera (Montreuil, pers. comm.).

The phylogeny obtained for Helictopleurini suggests a rapid radiation close to the origin of the tribe. The phylogeny provides support for a group of species belonging to the *giganteus* group, which is consistent with taxonomy based on morphological characters. Below we discuss the ecological context of adaptive radiations in dung beetles, and how this may elucidate the difference between the two tribes in Madagascar.

Adaptive radiations

Tropical forest and savanna dung beetle populations are universally limited by competition for resources (Hanski and Cambefort, 1991; Barbero et al., 1999), and the species that are present in the same community typically exhibit differences in their niches that facilitate coexistence (Krell et al., 2003; Krell-Westerwalbesloh et al., 2004). In Madagascar, local dung beetle communities have 20–30 species (Viljanen et al., in prep.), while the total number of species on the island is likely to be at least 300 (own unpubl. data). Therefore, there is extensive sorting of species into local communities, which is presumably affected by their ecological attributes. There is little doubt that the endemic dung beetles in Madagascar have undergone an adaptive radiation largely driven by intensive resource competition.

In Helictopleurini, the level of genetic divergence between pairs of species is smaller than in Canthonini as a tribe, but comparable with that in Canthonini clades, suggesting a more recent origin for Helictopleurini. Based on the sequence information of the encoding mitochondrial genes we estimated the ages of the Helictopleurini radiation and of the radiations of Canthonini clades using the absolute calibration of branch lengths of 2% divergence per million years (Myr) for insect mtDNA (Brown et al., 1979; Brower, 1994), which corresponds to the rate of nucleotide changes of 0.01 substitution/site/Myr. According to this calculation, Helictopleurini is 8.6 Myr old. This result is consistent with Paulian's (1987) view of recent colonization of Madagascar by Helictopleurini in the Miocene (24–5 Myr). The sister taxon of Helictopleurini is either Oniticellini or Onthophagini, and more likely the former (d'Orbigny, 1915; Koivulehto et al., in prep.). These taxa, together with Sisyphini and Coprini, comprise the Holarctic dung beetle fauna of Cambefort (Cambefort, 1991). Oniticellini are generally associated with large herbivore dung (Cambefort, 1991). In Madagascar, large herbivores have been represented in the past by large-bodied lemurs (up to 200 kg) and the pygmy hippopotami (up to 270 kg). Nowadays the surviving small lemurs (up to 7 kg) are the dominant mammalian group. The ancestors of lemurs arrived 62–65 Myr ago but the main lemur radiation is considered to have occurred about 10–15 Myr ago (Yoder and Yang, 2004). Many Helictopleurini species are specialized to use lemur dung, though there are also species with a more generalized diet and even species that are attracted primarily to carrion (Viljanen et al., in prep.). Our results for Helictopleurini are consistent with a rapid initial burst of speciation at the base of the radiation and lower extinction/speciation dynamics later on. Based on the timing of the Helictopleurini radiation and the presumed main radiation of lemurs, we suggest that Helictopleurini may have evolved in association with lemurs.

According to Paulian's (1987) hypothesis, Canthonini have colonized Madagascar during the Cretaceous (144–65 Myr). The estimated ages for the individual clades in Canthonini are 5.6, 9.3 and 12.3 Myr for *Sphaerocanthon*, *Apotolamprus* and *Arachnodes*, respectively. The sister clades *Sphaerocanthon* and *Apotolamprus* diverged 10.4 Myr ago (Fig. 2A, node 1), while the divergence between *Arachnodes* and the group *Sphaerochanthon/Apotolamprus* happened 14 Myr ago (Fig. 2A, node 2). These timings are in apparent conflict with Paulian's hypothesis of a Cretaceous colonization of Madagascar by Canthonini. Canthonini represents an old dung beetle taxon with a typical Gondwanian

distribution, being numerous in South America, southern Africa, Madagascar, south-east Asia and Australia (Lebis, 1953; Cambefort, 1991). Given this distribution, it is indeed quite possible that the ancestors of Canthonini were in Madagascar at the time of its separation with the African mainland, 160 Myr ago (de Wit, 2003). However, it is also possible that Madagascar has been subsequently colonized by Canthonini from mainland Africa, which could explain the presence of relatively recent taxa in Madagascar today (Fig. 2A,C). The ecology of Canthonini is different from that of Helictopleurini, with the majority of Canthonini species being either necrophagous or generalists, using both dung and carrion (Viljanen et al., in prep.). The necrophagous species use, among other things, the carcasses of abundant small mammals and other resources that have been present in Madagascar during all its history. Reconstruction of the colonization history of and radiation in Malagasy Canthonini requires a comprehensive global data set, including species from all the southern continents. We are presently collecting specimens and data to address these questions.

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Appendix 1

List of taxa studied with information on their collecting localities.

Genus	Species	Collecting site
Aleiantus	*A. frontalis* (Montreuil, 2003)	Madagascar, Ranomafana NP
	A. major (Paulian 1991)	Madagascar, Ranomafana NP
	A. viridicollis (Montreuil, 2005b)	Madagascar, Analamerana
Apotolamprus	*A. hanskii* (Montreuil, 2005a)	Madagascar, Mandena
	A. helenae (Montreuil, 2004)	Madagascar, Ranomafana NP
	A. quadrinotatus (Boucomont, 1937)	Madagascar, Ranomafana NP
	A. rubromaculatus (Künckel, 1887)	Madagascar, Ranomafana NP
Arachnodes	*A. andriai* (Paulian, 1976)	Madagascar, Baie de Baly
	A. antoetrae (Paulian, 1975)	Madagascar, Andringitra NP
	A. apotolamproides (Lebis, 1961)	Madagascar, Ranomafana NP
	A. balianus (Paulian, 1976)	Madagascar, Baie de Baly
	A. cuprarius (Fairmaire, 1889)	Madagascar, Ranomafana NP
	A. cyanescens (Brancksik, 1893)	Madagascar, Andavakoera
	A. dichrous (Paulian, 1976)	Madagascar, Andranomena
	A. kelifelyi (Paulian, 1976)	Madagascar, Kirindy Nord
	A. manaitrai kirindyensis (Montreuil, 2006)	Madagascar, Andranomena
	A. saprinoides (Fairmaire, 1889)	Madagascar, Montagne d'Ambre
	A. semipunctatus (Lebis, 1953)	Madagascar, Baie de Baly
	A. sicardi (Lebis, 1953)	Madagascar, Montagne d'Ambre
	Arachnodes sp. 1	Madagascar, Mandena
Pseudoarachnodes	*P. hanskii* (Montreuil, 2003)	Madagascar, Ranomafana NP
Sphaerocanthon	*S. bimaculatus* (Künckel, 1887)	Madagascar, Ranomafana NP
	S. dubitatus (Lebis, 1953)	Madagascar, Andasibe NP
	S. peyrierasi (Paulian, 1976)	Madagascar, Andavakoera
	S. viettei (Paulian, 1976)	Madagascar, Ranomafana NP
	S. hanskii (Montreuil, in press)	Madagascar, Analamerana
	S. occidentalis (Paulian, 1976)	Madagascar, Andranomena
Helictopleurus	*H. corruscus* (d'Orbigny, 1915)	Madagascar, Ranomafana NP
	H. fissicollis (Fairmaire, 1898)	Madagascar, Montagne d'Ambre
	H. fungicola (Fairmaire, 1899)	Madagascar, Daraina
	H. giganteus (Harold, 1869)	Madagascar, Italaviana
	H. marsyas (Olivier, 1789)	Madagascar, Andringitra NP
	H. multimaculatus (Lebis, 1960)	Madagascar, Ranomafana NP
	H. neoamplicollis (Krell, 2000)	Madagascar, Forêt de Mikea
	H. nicollei (Lebis, 1960)	Madagascar, Ambila
	H. obscurus (Lebis, 1960)	Madagascar, Masoala NP
	H. perrieri (Fairmaire, 1898)	Madagascar, Forêt de Mikea
	H. politicollis (Fairmaire, 1902)	Madagascar, Montagne d'Ambre

Appendix 1 *Continued*

Genus	Species	Collecting site
	H. purpuricollis (Lebis, 1960)	Madagascar, Arivonimamo
	H. rudicollis (Fairmaire, 1898)	Madagascar, Ranomafana NP
	H. semivirens (d'Orbigny, 1915)	Madagascar, Ranomafana NP
	H. sinuaticornis (Fairmaire, 1898)	Madagascar, Andringitra NP
	H. splendidicollis (Fairmaire, 1893)	Madagascar, Montagne d'Ambre
	H. unifasciatus (Fairmaire, 1901)	Madagascar, Marofandilia
Parascatonomus	*P. penicillatus* (Harold, 1879)	Laos, Asia

NP, National Park.

Appendix 2

List of taxa studied with information on their accession numbers (AN) to the NCBI public database. "–" indicates no AN available due to failing in the amplification of the specific locus.

Tribe	Genus	Species	Gene	AN GenBank
Canthonini	*Aleiantus*	*A. frontalis*	16S	DQ369607
			12S	–
			CytbB	DQ369412
			CytbK	DQ369390
			COIJN	–
			COITH	DQ369468
			COII	DQ369634
			18S	DQ369661
			28S	DQ369541
		A. major	16S	DQ369608
			12S	–
			CytbB	DQ369413
			CytbK	DQ369391
			COIJN	–
			COITH	DQ369469
			COII	DQ369635
			18S	–
			28S	DQ369542
		A. viridicollis	16S	DQ369609
			12S	DQ369356
			CytbB	DQ369414
			CytbK	DQ369392
			COIJN	DQ369467
			COITH	–
			COII	DQ369636
			18S	DQ369660
			28S	DQ369543
	Apotolamprus	*A. hanskii*	16S	DQ369610
			12S	DQ369357
			CytbB	DQ369415
			CytbK	DQ369393
			COIJN	DQ369586
			COITH	DQ369470
			COII	DQ369637
			18S	DQ369662
			28S	DQ369544
		A. helenae	16S	DQ369611
			12S	DQ369358
			CytbB	DQ369416
			CytbK	DQ369394
			COIJN	DQ369587
			COITH	DQ369471
			COII	DQ369638

Appendix 2 *Continued*

Tribe	Genus	Species	Gene	AN GenBank
			18S	DQ369663
			28S	DQ369545
		A. quadrinotatus	16S	DQ369612
			12S	DQ369359
			CytbB	–
			CytbK	DQ369395
			COIJN	DQ369588
			COITH	DQ369472
			COII	DQ369639
			18S	DQ369664
			28S	DQ369546
		A. rubromaculatus	16S	DQ369613
			12S	DQ369360
			CytbB	DQ369417
			CytbK	DQ369396
			COIJN	DQ369589
			COITH	DQ369473
			COII	DQ369640
			18S	DQ369665
			28S	DQ369547
	Arachnodes	*A. andriai*	16S	DQ369614
			12S	DQ369361
			CytbB	–
			CytbK	–
			COIJN	DQ369590
			COITH	DQ369474
			COII	DQ369641
			18S	DQ369666
			28S	DQ369548
		A. antoetrae	16S	DQ369615
			12S	DQ369362
			CytbB	DQ369418
			CytbK	DQ369397
			COIJN	–
			COITH	–
			COII	–
			18S	DQ369667
			28S	DQ369549
		A. apotolamproides	16S	
			12S	DQ369363
			CytbB	DQ369419
			CytbK	DQ369398
			COIJN	DQ369591
			COITH	DQ369476
			COII	DQ369642
			18S	DQ369669
			28S	DQ369550
		A. balianus	16S	DQ369617
			12S	DQ369364
			CytbB	DQ369420
			CytbK	DQ369399
			COIJN	DQ369592
			COITH	DQ369477
			COII	DQ369643
			18S	DQ369670
			28S	DQ369551
		A. cuprarius	16S	DQ369618
			12S	DQ369365
			CytbB	–
			CytbK	–
			COIJN	DQ369593
			COITH	DQ369478

Appendix 2 *Continued*

Tribe	Genus	Species	Gene	AN GenBank
			COII	DQ369644
			18S	DQ369671
			28S	DQ369552
		A. cyanescens	16S	DQ369619
			12S	DQ369366
			CytbB	DQ369421
			CytbK	DQ369400
			COIJN	DQ369594
			COITH	DQ369479
			COII	DQ369645
			18S	DQ369672
			28S	DQ369553
		A. dichrous	16S	DQ369624
			12S	–
			CytbB	–
			CytbK	–
			COIJN	–
			COITH	–
			COII	–
			18S	–
			28S	–
		A. kelifelyi	16S	DQ369620
			12S	DQ369367
			CytbB	DQ369422
			CytbK	–
			COIJN	DQ369595
			COITH	–
			COII	DQ369646
			18S	DQ369673
			28S	DQ369554
		A. manaitrai kirindyensis	16S	DQ369625
			12S	–
			CytbB	DQ369423
			CytbK	
			COIJN	DQ369598
			COITH	DQ369475
			COII	DQ369650
			18S	–
			28S	DQ369559
		A. saprinoides	16S	DQ369621
			12S	DQ369368
			CytbB	DQ369424
			CytbK	–
			COIJN	–
			COITH	–
			COII	DQ369647
			18S	DQ369675
			28S	DQ369555
		A. semipunctatus	16S	DQ369622
			12S	DQ369369
			CytbB	DQ369425
			CytbK	DQ369401
			COIJN	DQ369596
			COITH	DQ369481
			COII	DQ369648
			18S	DQ369676
			28S	DQ369556
		A. sicardi	16S	DQ369623
			12S	DQ369370
			CytbB	DQ369426
			CytbK	DQ369402
			COIJN	DQ369597
			COITH	DQ369482
			COII	DQ369649

Appendix 2 *Continued*

Tribe	Genus	Species	Gene	AN GenBank
			18S	DQ369677
			28S	DQ369557
		Arachnodes sp. 1	16S	DQ369626
			12S	DQ369371
			CytbB	DQ369427
			CytbK	DQ369404
			COIJN	DQ369599
			COITH	DQ369480
			COII	DQ369651
			18S	DQ369674
			28S	DQ369560
	Pseudoarachnodes	*P. hanskii*	16S	DQ369627
			12S	DQ369372
			CytbB	DQ369428
			CytbK	DQ369405
			COIJN	DQ369600
			COITH	DQ369483
			COII	DQ369652
			18S	DQ369678
			28S	DQ369561
	Sphaerocanthon	*S. bimaculatus*	16S	DQ369628
			12S	DQ369373
			CytbB	DQ369429
			CytbK	DQ369406
			COIJN	DQ369601
			COITH	–
			COII	DQ369653
			18S	DQ369681
			28S	DQ369562
		S. dubitatus	16S	DQ369629
			12S	DQ369374
			CytbB	DQ369430
			CytbK	DQ369407
			COIJN	DQ369602
			COITH	DQ369484
			COII	DQ369654
			18S	DQ369682
			28S	DQ369563
		S. peyrierasi	16S	DQ369630
			12S	DQ369375
			CytbB	DQ369433
			CytbK	DQ369408
			COIJN	DQ369603
			COITH	DQ369486
			COII	DQ369655
			18S	DQ369683
			28S	DQ369564
		S. viettei	16S	DQ369631
			12S	DQ369376
			CytbB	DQ369434
			CytbK	DQ369409
			COIJN	DQ369604
			COITH	DQ369487
			COII	DQ369657
			18S	DQ369684
			28S	DQ369565
		S. hanskii	16S	DQ369632
			12S	DQ369377
			CytbB	DQ369431
			CytbK	DQ369410
			COIJN	DQ369605
			COITH	–
			COII	DQ369658

Appendix 2 *Continued*

Tribe	Genus	Species	Gene	AN GenBank
			18S	DQ369679
			28S	DQ369566
		S. occidentalis	16S	DQ369633
			12S	DQ369378
			CytbB	DQ369432
			CytbK	DQ369411
			COIJN	DQ369606
			COITH	DQ369485
			COII	DQ369659
			18S	DQ369680
			28S	DQ369567
Helictopleurini	*Helictopleurus*	*H. corruscus*	16S	DQ369523
			12S	DQ369488
			CytbB	DQ369435
			CytbK	–
			COITH	DQ369450
			COII	–
			18S	DQ369568
			28S	DQ369505
		H. fissicollis	16S	DQ369524
			12S	DQ369489
			CytbB	–
			CytbK	DQ369379
			COITH	DQ369451
			COII	DQ369685
			18S	DQ369569
			28S	DQ369506
		H. fungicola	16S	DQ369525
			12S	DQ369490
			CytbB	DQ369436
			CytbK	–
			COITH	DQ369452
			COII	–
			18S	DQ369570
			28S	DQ369507
		H. giganteus	16S	DQ369526
			12S	DQ369491
			CytbB	DQ369437
			CytbK	–
			COITH	DQ369453
			COII	DQ369686
			18S	DQ369571
			28S	DQ369508
		H. marsyas	16S	DQ369527
			12S	DQ369492
			CytbB	DQ369438
			CytbK	DQ369380
			COITH	DQ369454
			COII	DQ369687
			18S	DQ369572
			28S	DQ369509
		H. multimaculatus	16S	DQ369528
			12S	DQ369493
			CytbB	DQ369439
			CytbK	DQ369381
			COITH	DQ369455
			COII	–
			18S	DQ369573
			28S	DQ369510
		H. neoamplicollis	16S	DQ369529
			12S	DQ369494
			CytbB	DQ369440
			CytbK	–
			COITH	DQ369456

Appendix 2 *Continued*

Tribe	Genus	Species	Gene	AN GenBank
			COII	DQ369688
			18S	DQ369574
			28S	DQ369511
		H. nicollei	16S	DQ369530
			12S	DQ369495
			CytbB	DQ369441
			CytbK	DQ369382
			COITH	DQ369457
			COII	DQ369689
			18S	DQ369575
			28S	DQ369512
		H. obscurus	16S	DQ369531
			12S	DQ369496
			CytbB	DQ369442
			CytbK	DQ369383
			COITH	DQ369458
			COII	DQ369690
			18S	DQ369576
			28S	DQ369513
		H. perrieri	16S	DQ369532
			12S	DQ369497
			CytbB	DQ369443
			CytbK	DQ369384
			COITH	DQ369459
			COII	DQ369691
			18S	DQ369577
			28S	DQ369514
		H. politicollis	16S	DQ369533
			12S	–
			CytbB	–
			CytbK	DQ369385
			COITH	–
			COII	DQ369692
			18S	DQ369578
			28S	DQ369515
		H. purpuricollis	16S	DQ369534
			12S	DQ369498
			CytbB	DQ369444
			CytbK	DQ369386
			COITH	DQ369460
			COII	DQ369693
			18S	DQ369579
			28S	DQ369516
		H. rudicollis	16S	DQ369535
			12S	DQ369499
			CytbB	DQ369448
			CytbK	DQ369387
			COITH	DQ369461
			COII	DQ369694
			18S	DQ369580
			28S	DQ369517
		H. semivirens	16S	DQ369536
			12S	DQ369500
			CytbB	–
			CytbK	DQ369388
			COITH	DQ369462
			COII	DQ369695
			18S	DQ369581
			28S	DQ369518
		H. sinuaticornis	16S	DQ369537
			12S	DQ369501
			CytbB	DQ369445
			CytbK	DQ369389
			COITH	DQ369463

Appendix 2 *Continued*

Tribe	Genus	Species	Gene	AN GenBank
			COII	DQ369696
			18S	DQ369582
			28S	DQ369519
		H. splendidicollis	16S	DQ369538
			12S	DQ369502
			CytbB	DQ369446
			CytbK	
			COITH	DQ369464
			COII	DQ369697
			18S	DQ369583
			28S	DQ369520
		H. unifasciatus	16S	DQ369539
			12S	DQ369503
			CytbB	DQ369447
			CytbK	–
			COITH	DQ369465
			COII	DQ369698
			18S	DQ369584
			28S	DQ369521
Onthophagini		*Parascatonomus penicillatus*	16S	DQ369540
			12S	DQ369504
			CytbB	DQ369449
			CytbK	–
			COITH	DQ369466
			COII	DQ369699
			18S	DQ369585
			28S	DQ369522

Appendix 3

Nucleotide frequencies at different codon positions calculated for all loci in Helictopleurini and Canthonini. The percentage of AT, χ^2 (chi-square) test for bias in nucleotide composition and *P*-values for the χ^2 test are shown.

Tribe	Codon position	T%	C%	A%	G%	A-T%	χ^2	*P*
Canthonini	16S	38.2	8.1	38.6	15.0	76.8	28.1	< 0.0001
	12S	37.4	16.1	38.4	8.2	75.8	26	< 0.0001
	Cytb (B + K)							
	First	44.0	10.3	38.5	7.15	82.5	42.3	< 0.0001
	Second	32.8	12.9	40.1	14.1	73	21.2	< 0.0001
	Third	30.9	20.7	31.5	16.8	62.4	6.3	0.01
	all	35.9	14.6	36.7	12.7	73.6	22.1	< 0.0001
	COI (TH + JN)							
	First	30.7	11.5	43.4	14.2	74.1	24	< 0.0001
	Second	32.5	21.1	27.9	18.4	60.5	4.4	0.04
	Third	41.4	14.7	35.4	8.5	76.8	28.1	< 0.0001
	all	34.9	15.7	35.6	13.7	70.5	16.8	< 0.0001
	COII							
	First	38.8	17.0	34.1	10.1	72.9	20.3	< 0.0001
	Second	45.3	8.8	41.0	4.8	86.3	53.3	< 0.0001
	Third	33.8	14.5	35.8	15.9	69.6	15.2	< 0.0001
	all	39.3	13.5	37.0	10.3	76.3	28.1	< 0.0001
	18S							
	First	23.6	21.1	26.5	28.7	50.1	0.200E-01	0.9
	Second	19.8	23.5	25.1	31.6	44.9	1.2	0.3
	Third	23.1	26.0	26.2	24.6	49.3	0.200E-01	0.9
	all	22.2	23.6	25.9	28.3	48.1	0.1	0.7
	28S							
	First	18.6	23.3	31.2	26.9	49.8	0.200E-01	0.9
	Second	10.0	27.9	24.5	37.6	34.5	9.6	0.02
	Third	21.0	26.1	18.6	34.3	39.6	4.4	0.04
	all	16.5	25.8	24.7	33	41.2	2.9	0.09
Helictopleurini	16S	38.9	7.7	39.6	13.7	78.5	32.5	< 0.0001
	12S	40.0	13.9	38.8	7.3	78.8	32.5	< 0.0001
	Cytb (B + K)							
	First	29.6	18.55	35.0	16.85	64.6	8.4	0.004
	Second	36.5	18.6	34.9	9.95	71.4	18.5	< 0.0001
	Third	39.1	8.4	39.0	13.35	78.1	32.5	< 0.0001
	all	35.1	15.2	36.3	13.4	71.4	18.5	< 0.0001
	COI (TH)							
	First	19.2	14.6	45.0	21.3	64.2	8.42	0.004
	Second	32.6	28.9	27.6	10.8	60.2	11.7	0.001
	Third	46.1	1.8	46.3	5.7	92.4	72.3	< 0.0001
	all	32.6	15.1	39.6	12.6	72.2	20.3	< 0.0001
	COII							
	First	30.6	14.7	39.4	15.3	70.0	16.0	< 0.0001
	Second	39.9	18.8	29.6	11.7	69.5	15.2	< 0.0001
	Third	45.6	7.2	42.7	4.4	88.3	59.3	< 0.0001
	all	38.7	13.6	37.2	10.5	75.9	26.0	< 0.0001
	18S							
	First	22.9	24.8	24.5	27.8	47.4	0.3	0.6
	Second	23.0	19.0	29.3	28.6	52.3	0.3	0.6
	Third	21.5	25.4	25.1	28.0	49.6	0.200E-01	0.9
	all	22.5	23.1	26.3	28.1	48.8	0.1	0.7
	28S							
	First	19.0	2.07	31.6	28.8	50.6	0.200E-01	0.9
	Second	14.5	25.1	24.4	36.0	38.9	5.3	0.02
	Third	21.1	25.4	22.7	30.7	43.8	1.7	0.2
	all	18.2	23.7	26.2	31.9	44.4	1.2	0.3

Appendix 4

Transformations calculated for all mitochondrial genes at different codon positions and for the pooled codon positions (all) in Helictopleurini and Canthonini (Fig. 5). Ts denotes transitions, Tv transversions.

Appendix 4 *Continued*

The fit models of nucleotide substitution and likelihood scores calculated for all loci using Modeltest 3.04 as implemented in PAUP.

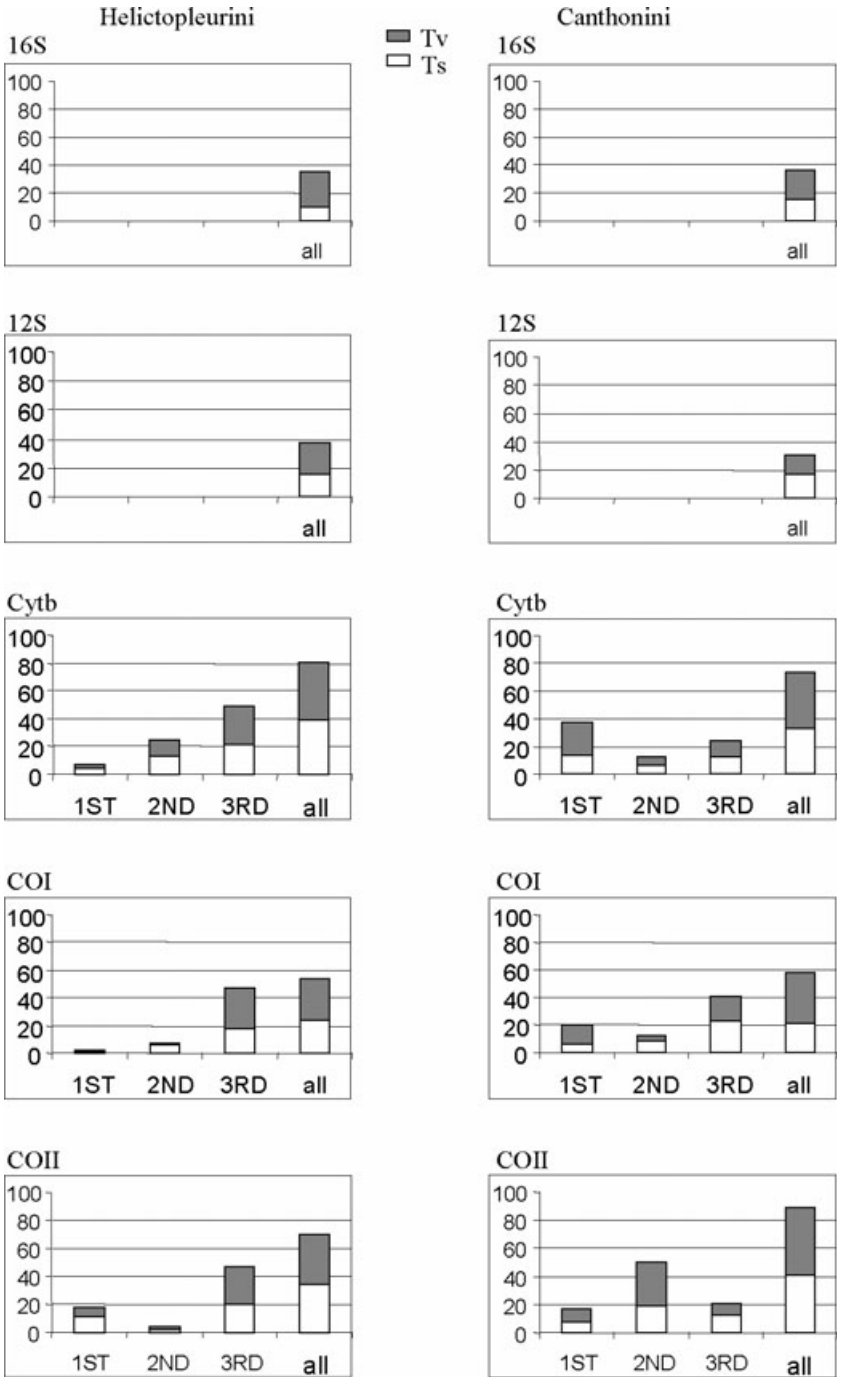

Fig. 5. Transformations calculated for all mitochondrial genes at different codon positions and for the pooled codon positions (all) in Helictopleurini and Canthonini. Ts denotes transitions, Tv transversions.

Tribe	Locus	Model	–lnL score
Canthonini	16S	GTR + I + G	2255.4
	12S	TrN + I + G	1971.4
	CytbB	GTR + I + G	2957.2
	CytbK	GTR + I + G	5491.4
	COI JN	GTR + I + G	3024.1
	COI TH	GTR + I + G	3247.67
	COII	GTR + I + G	5305.59
	18S	TrNef	1296.8
	28S	TrNef + I + G	728.6
Helictopleurini	16S	F_{81} + G	1843.5
	12S	TVM + G	1771.98
	CytbB	GTR + I + G	1985.4
	CytbK	GTR + I + G	3459.8
	COITH	GTR + I + G	2529.8
	COII	GTR + I + G	3274.1
	18S	JC	1261.9
	28S	K80 + I	554.1

Appendix 5

AT richness and transition/transversion ratios (Ts/Tv) in individual genes for Helictopleurini and Canthonini. Published data for other groups of insects are also reported.

Data set	No.	Locus	A + T percentage	Ts/Tv	Reference
Canthonini (Scarabaeidae)	27	16S	76.8	0.7	Present paper
		12S	75.8	1.2	
		Cytb	73.6	0.8	
		COI	70.5	0.6	
		COII	76.3	0.8	
Helictopleurini (Scarabaeidae)	17	16S	78.5	0.4	Present paper
		12S	78.8	0.7	
		Cytb	71.4	0.95	
		COI	72.2	0.8	
		COII	75.9	0.9	
Lice (Phthiraptera)	150	COI	62.4	1.63	(Johnson et al., 2003)
Aphids (Aphidae)	100	COI	77.2	0.9	(Clark et al., 2000)
		COII	79.8	1.01	
Hymenoptera (Aphidae)	28	COI	79.3		(Bull et al., 2003)
Treehoppers (Membracidae)	112	COI	70.2	1.77	(Lin et al., 2004)
		COII	75.8	1.93	
		12S	73.1	0.70	
Gall-inducing thrips (Thripidae)	21	COI	72.6	1.7	(Morris et al., 2001)
Stalk-eyed flies (Diopsidae)	33	COII	72.6	1.5	(Baker et al., 2001)
Nymphalid butterflies (Nymphalidae)	22	COI and COII	76.3	1.06	(Brower and DeSalle, 1998)
Nymphalid butterflies (Nymphalidae)	54	COI	69	1.27	(Monteiro and Pierce, 2001)
		COII	76	1.14	
Swallowtail butterflies (Papilionidae)	37	COI and COII	74	0.89	(Caterino et al., 2001)
Swallowtail butterflies (Papilionidae)	23	COI and COII	73.5	1.16	(Reed and Sperling, 1999)
Swallowtail butterflies (Papilionidae)	23	COI and COII	73.5		(Caterino and Sperling, 1999)
Nymphalid butterflies (Nymphalidae)	25	COI and COII	80		(Beltran et al., 2002)
Halictine bees (Halictidae)	48	COI	74	1.96	(Danforth et al., 2003)
Carpenter bees (Xylocopinae)	22	COI	77.9	1.24	(Leys et al., 2000)
		Cytb	80.2		
Bark beetles (Scolytinae)	44	COI	67.4	1.2	(Cognato and Vogler, 2001)
		16S		0.54	
Carabid beetles (Carabidae)	33	16S	75.5	2.74	(Sota et al., 2001)

Appendix 6 *Continued*

Data set	No.	Locus	A + T percentage	Ts/Tv	Reference
Cicindela (Cicindelidae)	51	COI, COIII, Cytb and 16S	75.9		(Pons et al., 2004)
Drosophila (Drosophilidae)	Ca 15	COI	70.9		(Moriyama and Powell, 1997)
Spittlebugs (Cercopidae)	1	Complete mtDNA	77		(Stewart and Beckenbach, 2005)

No., number of species.